Cancer Chemother Pharmacol (1988) 21: 274–280

Cancer Chemotherapy and Pharmacology

Selectivity of the anthracyclines for negatively charged model membranes: role of the amino group*

Thomas G. Burke[2], Alan C. Sartorelli[2], and Thomas R. Tritton[1]

[1] Department of Pharmacology and Vermont Regional Cancer Center, University of Vermont Medical School, Burlington, VT 05405, USA
[2] Department of Pharmacology and Developmental Therapeutics Program, Comprehensive Cancer Center, Yale University School of Medicine, New Haven, CT 06510, USA

Summary. The equilibrium-binding affinities of six adriamycin analogues and four daunomycin derivatives for negatively charged dimyristoyl phosphatidylcholine/dimyristoyl phosphatidic acid (DMPC/DMPA) small unilamellar vesicles are compared with values for electroneutral DMPC liposomes. Binding of the daunomycin series to negatively charged dimyristoyl phosphatidyl glycerol (DMPG) vesicles was also examined. Under physiological conditions of pH and ionic strength, substitution of the amino group of adriamycin or daunomycin resulted in a reduced affinity for negatively charged bilayers, even if the substituent enhanced the degree of ionization of the amine. Decreasing the ionic strength increases the binding affinity for acidic membranes but decreases the drug affinity for neutral membranes. We propose that the electrostatic bond of the phosphate-amino group that has been shown to exist between anthracyclines and phosphatidic acid is sterically destabilized by substitution of the amino group. The results are consistent with a mode of anthracycline binding to negatively charged membranes which is driven by hydrophobic and electrostatic considerations but is destabilized by steric bulk at the amino group. The data also provide insight into the design of new anthracyclines with high membrane affinities and reduced uptake; such directed interaction with plasma membranes may enhance antineoplastic potential while reducing cardiac toxicity.

Introduction

The anthracyclines adriamycin and daunomycin are widely used antineoplastic agents that have therapeutic activity against a broad variety of human cancers [22]. The interactions of these agents with membranes are of interest for several reasons. The classic paradigm for anthracycline activity has been direct interference with DNA function; thus, in order for anthracyclines to elicit cellular responses by interacting with DNA or with other intracellular components, the drug molecules must first interact with and then successfully traverse membranes. Another compelling reason for investigating anthracycline-membrane interactions is provided by the recent observations showing that polymer-immobilized congeners exert their cytotoxic activity against neoplastic cells solely by a cell surface interaction [29, 30, 35, 37, 39]. Finally, specific interactions between anthracycline molecules, and negatively charged membrane lipids have been implicated in the toxicity of these drugs to cardiac tissues [13, 17, 20, 38].

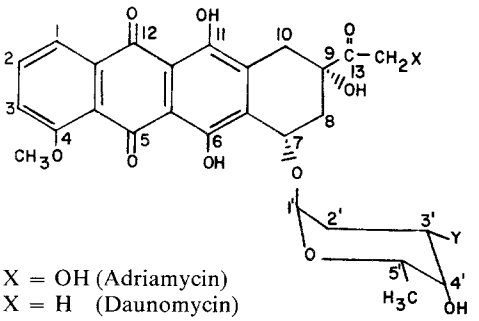
We have recently reported on fluorescence studies on the interactions of anthracyclines with neutral phospholipid bilayers, as represented by small unilamellar phosphatidylcholine vesicles [5–7]. A systematic study using several daunomycin analogues, all of which differed from the parent molecule by a single structural change in the aglycone portion of the molecule, showed that a strong correlation existed between drug hydrophobicity and both membrane affinity [6] and membrane penetration [7]. These findings are consistent with the notion that anthracycline associations with neutral phospholipid bilayers (both fluid-phase and solid-phase) are dominated by hydrophobic interactions between the aglycone portion of the anthracycline molecule and the hydrocarbon interior of the membrane.

However, neutral membranes are probably not representative of the real biologic situation experienced by adriamycin and daunomycin. Most membranes bear a net (usually negative) charge. It is therefore important to ascertain how these drugs interact with membranes bearing an overall surface charge. Further motivation for extending the previous studies has resulted from the demonstration that adriamycin binds preferentially to negatively charged membranes rather than to electroneutral membranes; electrostatic interactions between the negatively charged phosphate and the partially positively charged amino group of the sugar moiety enhanced the stability of the drug-membrane complex [13, 17, 19, 20, 38].

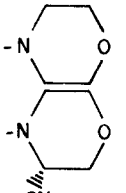
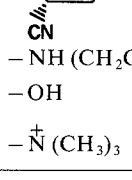
Table 1 shows the molecular structures of a set of adriamycin and daunomycin derivatives chosen to exhibit a wide range of structure and function. We employed fluorescence anisotropy titration and evaluated the manner

* This research was supported in part by U. S. Public Health Service Grants CA-02817, CA-44729, and CA-01088 from the National Cancer Institute and by a grant from the American Cancer Society (CH-392).
Thomas R. Tritton was the recipient of a Research Career Development Award (CA-00684) and Thomas G. Burke was supported by an NIH Training Grant (CA-09085)
Offprint requests to: T. R. Tritton
Abbreviations: DMPC, L-α-dimyristoyl phosphatidylcholine; DMPG, L-α-dimyristoyl phosphatidyl glycerol; DMPA, L-α-dimyristoyl phosphatidic acid; TLC, thin-layer chromatography; PBS, phosphate-buffered saline containing 8 m*M* Na_2HPO_4, 1 m*M* KH_2PO_4 and the specified amount of KCl(pH 7.4); T_m, gel to liquid-crystalline phase transition temperature; DMPG, L-α-dimyristoyl phosphatidyl glycerol; SUVs, small unilamellar vesicles

Table 1. Structures of anthracycline derivatives



X = OH (Adriamycin)
X = H (Daunomycin)

Derivative	Y
Parent	$-NH_2$
N,N-Dimethyl	$-N(CH_3)_2$
Morpholino	
Cyanomorpholino	
N-(Cyanomethyl)	$-NH(CH_2CN)$
3′-Hydroxy	$-OH$
N,N,N-Trimethyl	$-\overset{+}{N}(CH_3)_3$

in which substitution of the anthracycline amino group affected drug selectivity for negatively charged membranes under physiological conditions. The data obtained are consistent with a mode of anthracyclines binding to negatively charged membranes that is driven by hydrophobic and electrostatic considerations, but which is destabilized by steric bulk at the amino group.

Materials and methods

Chemicals. Adriamycin, daunomycin, *N,N*-dimethyl adriamycin, and *N,N*-dimethyl daunomycin were the gift of Dr Leonard Kedda of the Division of Cancer Treatment, National Cancer Institute. 3'-Deamino-3'-(4-morpholinyl)-adriamycin, 3'-deamino-3'-(4-morpholinyl)daunomycin 3'-deamino-3'-(3-cyano-4-morpholinyl)adriamycin, 3'-deamino-3'(3-cyano-4-morpholinyl)-daunomycin, *N,N,N*-trimethyl adriamycin, and *N*-(cyanomethyl)adriamycin were generously provided by Dr Edward M. Acton of M.D. Anderson Hospital and Tumor Institute, Houston, Tex. 7-O-(2,6-Dideoxy-α-L-lyxohexopyranosyl)adriamycinone (or 3'-hydroxyadriamycin) was the gift of Dr Derek Horton of The Ohio State University, Columbus, Ohio. *N,N,N*-Trimethyl daunomycin was synthesized from daunomycin by the unpublished method of G. Tong, SRI International, Melno Park, California. This synthesis involved the dropwise addition of 1.4 mg $NaOCH_3$ in 40 µl methanol to a stirred solution of 15 mg daunomycin in 300 µl methanol cooled in an ice bath. The solution was stirred and diluted with 160 µl chloroform, after which 14 µl methyl iodide and 6.8 mg $NaHCO_3$ were added. The mixture was stirred at ambient temperature in the dark for 3 days, after which another 14 µl methyl iodide was added and the reaction continued for 2 additional days. Insoluble $NaHCO_3$ was removed by filtration, and the aqueous mixture was washed with 2×5 ml chloroform to remove trace amounts of aglycone. The aqueous mixture was then frozen and lyophilized. Thin-layer chromatography (TLC) analysis on silica gel using a solvent system of chloroform-methanol-water (40:10:1, v/v) showed that all of the daunomycin had been converted to its quaternary form.

All the above anthracyclines were in the hydrochloride form except for the *N*-(cyanomethyl) and cyanomorpholino derivatives, which were in the free base form. The anthracyclines were used without further purification, since TLC analysis showed their purity to be greater than 98%. Stock solutions of the drugs were prepared in methanol or ethanol and stored in the dark at −20° C. The following absorption wavelengths and extinction coefficients for methanol solutions were used to calibrate drug stocks: adriamycin, 480 nm, 12200 M^{-1} cm^{-1} [9]; daunomycin, 478 nm, 12100 M^{-1} cm^{-1} [25]; *N,N*-dimethyldaunomycin, 478 nm, 12500 M^{-1} cm^{-1} [36]; 3'-deamino-3'-(4-morpholinyl)adriamycin, 479 nm, 12700 M^{-1} cm^{-1} [2]; 3'-deamono-3'-(4-morpholinyl)daunomycin, 479 nm, 11800 M^{-1} cm^{-1} [25]; 3'-deamino-3'-(3-cyano-4-morpholinyl)daunomycin, 479 nm, 12300 M^{-1} cm^{-1} [2]; 3'-deamino-3'-(3-cyano-4-morpholinyl)adriamycin, 479 nm, 12400 M^{-1} cm^{-1} [2]; *N*-(cyanomethyl) adriamycin, 479 nm, 12500 M^{-1} cm^{-1}; and 3'-hydroxyadriamycin, 480 nm, 12100 M^{-1} cm^{-1}. The extinction coefficient of a quaternized anthracyclines was assumed to be the same as that of its parent.

L-α-Dimyristoyl phosphatidylcholine (DMPC), L-α-dimyristoyl phosphatidic acid (DMPA), and L-α-dimyristoyl phosphatidyl glycerol (DMPG) were obtained from Avanti Biochemicals (Birmingham, Ala) and were used without further purification. All other chemicals were reagent grade.

Vesicle preparation. DMPC, DMPG, and DMPC/DMPA (molecular ratio of 2:1) master stock solutions in chloroform were prepared, from which 9.8×10^{-5} mol lipid was placed in a series of glass sonication tubes. The chloroform was removed by a stream of nitrogen, resulting in the formation of a lipid film on the tube wall. Residual chloroform was removed by placing the samples in a vacuum desiccator for 24 h, and the samples were then stored in the dark at −20° C. Lipid dispersions were prepared on the day of an experiment by the addition of 7.0 ml buffer to the tubes containing the dried lipid film (total lipid concentration of 1.4×10^{-2} *M*), followed by Vortex mixing for 5–10 min above the gel to liquid-crystalline phase transition temperature (T_m). Small unilamellar vesicles were than prepared by the method of Poste et al. [28]. The lipid dispersions were subjected to ultrasound with a Laboratories Supplies Company (Hicksville, NY) bath-type sonicator at approximately 50° C for 30 min or until no further optical clearing of the solution was observed. The pH values of solution were checked and readjusted when necessary to pH 7.4 before and during sonication to compensate for pH changes due to the ionization of the DMPG or DMPA molecules. TLC analysis on silica gel using a solvent system of chloroform-methanol-acetic acid-water (25:15:4:2, v/v) revealed that no decomposition of the lipids had occurred during sonication. The suspensions were annealed at 50° C for 30 min prior to use.

Fluorescence instrumentation. Fluorescence measurements were obtained using an SLM model 4800 subnanosecond spectrofluorometer equipped with EMI Industrial Electronics Ltd. (Ruislip, England) 9816A phototubes. Steady-state fluorescence intensity and anisotropy measurements were obtained as previously described [6, 7]. Fluorescence measurements on anthracycline samples were conducted using an excitation wavelength of 470 nm and a bandpass of 4 nm, two 500-nm short pass filtres (Melles Griot) in the excitation beam to reduce the transmission of stray light from the monochromator, and a 550-nm long pass filter (Schott) for each emission channel to isolate fluorescence from scattered light. All experiments were conducted in 1-cm quartz cuvettes. The background fluorescence or scatter from unlabeled lipids or from solvents was typically less than 2% of the total intensity, and anisotropy and intensity measurements were corrected for the background signal.

Calorimetry. Differential scanning calorimetry was performed on a Perkin-Elmer (Norwalk, CT) model DSC-7 calorimeter at a scan rate of 1° C/min. Each sample pan consisted of a three-part (pan, cover, o-ring) hermetically sealed stainless steel assembly (Perkin-Elmer, Norwalk, CT). All experiments were conducted using 40-μl samples, the reference cell containing the same weight of water as the sample cell. The typical weight of lipid in each experiment was 4 mg. A T_m value refers to the peak temperature of the transition.

To verify that the DMPC, DMPC/DMPA, and DMPG liposomes were in their liquid-crystalline state at 37° C, the temperature of the binding experiments, the following calorimetric experiments were performed. A sample of DMPC SUVs was placed in the calorimeter, quickly cooled to 15° C and scanned upward in temperature. SUVs are known to be unstable below the T_m because they fuse to slightly larger unilamellar vesicles [11, 14, 26, 27, 33]; consequently, the period of time spent below the T_m was kept to a minimum. The endotherm of the DMPC SUVs was centered at 20° C, in reasonable agreement with the previously reported value of 20.8° C determined at a scan rate of 2.5° C/min [14]. The DMPG SUVs exhibited a T_m value of 25° C, while the DMPA SUVs exhibited a value of 52° C. Recent studies by Kouaouci et al. [21] and Graham et al. [18] have shown that DMPC and DMPA are completely miscible in the absence of calcium. To confirm that the DMPC-DMPA (2:1) SUVs used in our experiments were stable at 37° C, a sample was transferred to the calorimeter and incubated at 37° C for 2 h, the time course of the fluorescence experiments. Scanning downward in temperature showed an exothermic event for the DMPC/DMPA (2:1) SUVs at 27° C. A similar calorimetric trace was observed for the DMPC/DMPA vesicles without the 2-h incubation at 37° C, indicating that these liposomes were stable in their liquid-crystalline state over the time period of interest.

Equilibrium binding measurements. Fluorescence anisotropy titration was used to determine the concentrations of free and bound drug in liposome samples containing a total drug concentration of 2×10^{-6} *M* and varying lipid concentrations by a method previously described [5, 6]. All experiments were conducted in siliconized glass tubes. The overall association constants are defined as:

$$K = [A_F] / [A_F][L], \quad (1)$$

where $[A_F]$ represents the concentration of bound drug, $[A_B]$ represents the concentration of free drug, and [L] represents the total lipid concentration in the vesicle suspension. The K values were determined from the slope of a double-reciprocal plot [5, 6].

Results

Dependence of binding affinity on anthracycline structure and membrane charge

Fluorescence anisotropy titration was used to determine the concentrations of free and liposome-bound drug at a total drug concentration of 2×10^{-6} *M* using a method which has been previously described [5, 6]. At this drug concentration, the experiments were free of antracycline self-association in solution; this has been shown to occur at drug concentrations in excess of 1×10^{-5} *M* [4, 10, 23, 24]. In addition, by gathering data under conditions where the concentration of free lipid was in great excess over the concentration of bound drug, we have avoided potential complications due to anthracycline self-association on the surface of liposomes, which has been shown to occur under conditions approaching saturation [5]. The linearity of the double-reciprocal plots used to determine the overall association constants (data not shown) provided evidence that the experiments were free of contamination due to the anthracycline self-association phenomenon and also indicated that fluorophore binding was adequately described by Eq. 1.

The overall association constants for the interaction of anthracyclines with neutral and negatively charged membranes, summarized in Table 2, were determined under near-physiological conditions of pH and ionic strength. At 37° C, the DMPC, DMPC/DMPA, and DMPG small unilamellar vesicles were all in their liquid-crystalline state (see Materials and methods). Thus, the binding data presented in Table 2 allow for a straightforward assessment of: (1) anthracycline selectivity for lipid bilayers containing negatively charged phosphate headgroups, and (2) the manner in which anthracycline selectivity for negatively charged membranes is affected by substitution of the amino group.

Of the derivatives studied, the parent morpholino and *N,N*-dimethyl congeners contain partially ionized amino groups at physiological pH. The pK_a of the amino group of adriamycin has been reported to be 8.2 [32], and the pK_a values of the *N,N*-dimethyl and morpholino derivatives are thought to be higher because of the presence of the electron-releasing groups [1]. The *N*-cyanomethyl and cyanomorpholino derivatives have significantly reduced pK_a values [2, 8], and these drugs are non-ionized at physiological pH. The *N,N,N*-trimethyl derivatives are fully ionized. Accordingly, the degree of ionization of the anthracycline congeners at physiological pH decreases in the following order: *N,N,N*-trimethyl > *N,N*-dimethyl and morpholino > parent > *N*-cyanomethyl and cyanomorpholino.

Comparison of the binding data within a given series of congeners demonstrates the manner in which substitution at the anthracycline amino group altered membrane binding. For both the adriamycin and daunomycin series, the nonbasic cyanomorpholino analogue had the highest affinity for electroneutral DMPC bilayers. *N*-Cyanometh-

Table 2. Overall association constants [K(M^{-1})] for anthracyclines interacting with neutral und negatively charged model membranes under physiological conditions[a]

Derivate	Association constant				
	Adriamycin series		Daunomycin series[b]		
	DMPC	DMPC/DMPA	DMPC	DMPC/DMPA	DMPG[c]
Cyanomorpholino[d]	260	340	1300	1100	1400
N-Cyanomethyl	200	260	–	–	–
Parent[d]	200	2700	1100	6500	15000
3′-Hydroxy	190	200	–	–	–
Morpholino[d]	160	450	950	1250	3800
N,N-Dimethyl[d]	90	600	450	2700	8300
N,N,N-Trimethyl	60	550	350	1700	4000

[a] Sonicated DMPC liposomes were used to represent electroneutral membranes. Sonicated DMPC/DMPA (2:1) and DMPG vesicles were used to represent negatively charged membranes. All experiments were conducted in PBS buffers, pH 7.4, at 37°C. Fluorescence anisotropy titration was used to determine the concentrations of free and bound drug at a total drug concentration of 2×10^{-6} *M*, as previously described [5,6]. Overall association constants were determined from the inverse of the slope of a double-reciprocal plot under conditions where the concentration of free lipid was in great excess over the concentration of bound drug. The best linear-least-sqares fit of the binding data for a minimum of ten samples of varying lipid concentration was obtained, from which the overall association was determined. Each of the double reciprocal plots was linear, indicative of a homogenous population of bound drug

[b] The *N*-cyanomethyl and 3′-hydroxy derivatives of daunomycin were not available for this study

[c] Anthracycline binding to DMPG liposomes was limited to the daunomycin series because of the large quantities of lipid required for this type of binding assay

[d] Affinities of these compounds for DMPC liposomes at 27.5°C have been reported elsewhere [8]

yladriamycin and 3'-hydroxyadriamycin, two other nonbasic anthracyclines, were found to have similar equilibrium binding affinities for DMPC bilayers as adriamycin. The morpholino and *N,N*-dimethyl derivatives of adriamycin and daunomycin were shown to have reduced affinities for DMPC bilayers. The fully ionized *N,N,N*-trimethyl analogues had the lowest DMPC affinities in each anthracycline series. Thus, increased ionization of the anthracycline amino group generally resulted in reduced affinity for neutral membranes. These results in conjunction with those of a previous investigation [6] indicate that a hydrophobic aglycone and a nonbasic aminosugar promote anthracycline binding for neutral membranes.

Cyanomorpholinoadriamycin, *N*-cyanomethyladriamycin, 3'-hydroxyadriamycin and cyanomorpholinodaunomycin all showed a relatively small change (30% or less) in binding which occurred upon the incorporation of negatively charged DMPA into the DMPC bilayer. In contrast to these adriamycin and daunomycin analogues with nonbasic aminosugars, the parent, morpholino, *N,N*-dimethyl and *N,N,N*-trimethyl anthracyclines all showed significantly enhanced binding (3 to 13-fold) upon DMPA incorporation into the DMPC bilayer. The parent drugs had by far the highest affinities for DMPC/DMPA bilayers within each anthracycline series, followed by the *N,N*-dimethyl, *N,N,N*-trimethyl, and morpholino derivatives. It should be noted that although the presence of an ionizable amino group appears to be an important factor in anthracycline selectivity for the negatively charged DMPC/DMPA membranes, a strict correlation between the degree of ionization of the amino group, and DMPC/DMPA affinity was not observed. This is not surprising since a single structural variable would not be expected to dominate a complex binding interaction. The finding that each daunomycin derivative exhibited a higher affinity for DMPC/DMPA bilayers than its corresponding adriamycin analogue indicates that in addition to an ionizable amine, the presence of a relatively hydrophobic aglycone also promotes anthracycline binding to negatively charged membranes.

The binding of the daunomycin series to DMPG bilayers is also summarized in Table 2. The daunomycin analogue exhibited the same order of affinity for DMPG bilayers as for DMPC/DMPA bilayers. Congeners with a partially or fully ionized amino group at pH 7.4 exhibited higher affinities for the homogenous DMPG bilayers than for the DMPC/DMPA mixed lipid bilayers. This finding was predictable, since the DMPG bilayers have a higher negative surface potential.

Dependence of anthracycline binding on ionic strenght and pK_a

We have previously shown that daunomycin binding to neutral fluid-phase DMPC bilayers increased with increasing ionic strength [7]. This finding was taken as evidence that ionic interactions between the anthracycline aminosugar and the polar head groups of the bilayer were not important considerations in binding; rather hydrophobic associations between the aglycone portion of the drug molecule and the hydrocarbon interior of the membrane appeared to be dominant. To investigate the contribution of ionic interactions in anthracycline binding to negatively charged membranes, we have examined the ionic strength dependence of morpholinodaunomycin and cyanomorpholinodaunomycin binding to mixed DMPC/DMPA bilayers and homogenous DMPC bilayers at 37° C. Morpholinodaunomycin and cyanomorpholinodaunomycin were chosen because these derivatives are structurally very similar but have been shown to have drastically different pK_a values [8]. The pK_a of morpholinodaunomycin is thought to be greater than 8.2, whereas cyanomorpholinodaunomycin, with a nitrile group adjacent to the amino moiety, has a pK_a of about 2 [2].

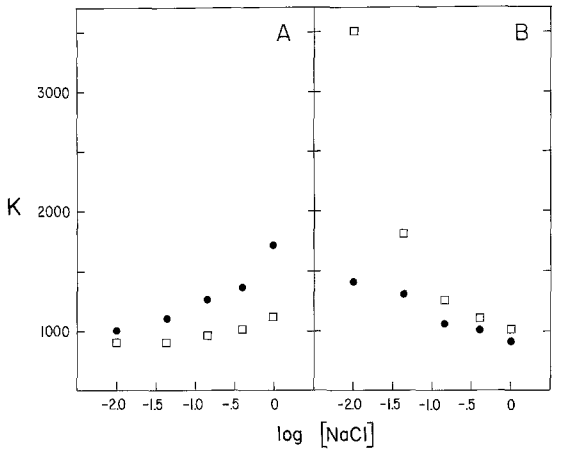


Fig. 1A, B. Ionic strength dependence of the overall binding affinities of morpholinodaunomycin (□) and cyanomorpholinodaunomycin (●) for **A** electroneutral DMPC and **B** negatively charged DMPC/DMPA (2:1) liposomes. Liposomes were prepared in PBS buffer, pH 7.4, containing the indicated concentration of NaCl. Studies were conducted at 37° C where both types of vesicles were in their liquid-crystalline phase. Binding isotherms were constructed using a fluorescence anisotropy titration method where a fixed drug concentration (2×10^{-6} *M*) was titrated with solutions of increasing lipid concentration as described in Materials and methods

Figure 1 depicts the dependence of anthracycline binding on ionic strength and pK_a. Panel A shows that the binding of both drugs to neutral DMPC bilayers increased with increasing ionic strength. In contrast, the binding of both drugs decreased with increasing ionic strength for negatively charged DMPC/DMPA bilayers, with the binding of the partially ionized morpholino derivative being reduced to a much greater degree with the increase in ionic strength.

Discussion

Effects of substitutions at the amino group on anthracycline selectivity for negatively charged membranes

Previous studies of adriamycin interactions with negatively charged membranes containing cardiolipin or phosphatidic acid have provided evidence for two types of binding environments for the drug, one relatively exposed and the other more deeply buried in the membrane [15, 19, 20], the deeply buried one being the high-affinity site [19]. To avoid complications in the analysis of fluorescence binding data due to multiple types of sites or anthracycline self-association at the surface of liposomes (5) we chose experimental conditions where the concentration of free lipid was in great excess over that of bound drug. Thus, the equilibrium binding affinities listed in Table 2 reflect anthracycline association with only high-affinity sites. These K values provide for a simple and convenient comparison of anthracycline association with neutral and negatively charged membranes.

By analyzing changes in the circular dichroism spectrum of the drug, Henry et al. [19] have recently demonstrated that adriamycin's high affinity site in negatively charged membranes involves: (1) an electrostatic bond between the amino group of the sugar moiety and the ionized phosphate group of cardiolipin or phosphatidic acid, and (2) hydrophobic associations between the aglycone portion of the drug molecule and the bilayer interior. Consistent with a binding phenomenon dependent upon electrostatic interactions, we have demonstrated in this report that anthracyclines with partially or fully ionized amino groups at physiological pH exhibited high affinities for negatively charged membranes and that the binding of one such analog, morpholinodaunomycin, was diminished significantly by increasing ionic strength. Also consistent with the previous findings of Henry et al. [19] were the observations that each daunomycin derivative had a higher affinity for negatively charged DMPC/DMPA bilayers than its corresponding adriamycin analogue, indicating that favorable hydrophobic interactions also contribute to drug binding.

In addition, our results clearly demonstrate that substitution of the amino group of adriamycin or daunomycin results in a reduced affinity for negatively charged bilayers, even if the substituent enhances the degree of ionization of the amino group. For example, the *N,N,N*-trimethyl derivatives, which carry a full positive charge, exhibited a four- to five-fold reduction in binding to negatively charged DMPC/DMPA or DMPG bilayers relative to their respective parent. To explain these observations, we propose that the electrostatic bond, of the phosphate amino group, which has been shown to exist between anthracyclines and phosphatidic acid [19], is sterically destabilized by amine substitution. Thus, our data are consistent with a mode of anthracycline binding to negatively charged membranes that is driven by both hydrophobic and electrostatic factors but which is destabilized by steric bulk at the amino group. By contrast, anthracycline binding to DNA appears to be dominated largely by ionic interactions between nucleoside phosphates and the sugar amino group [7, 9, 10]. Thus, there are basic differences between the physicochemical forces which govern binding to membranes and DNA. This in turn suggests that appropriate structural modification can differentially influence binding to these two types of sites.

Implications of anthracycline selectivity for negatively charged membranes in cellular transport

The exact mechanism by which anthracyclines traverse the surface membrane of cells is uncertain, although much of the available data indicates that the cellular accumulation of these agents occurs by passive diffusion of the electroneutral form of the drug molecule through the lipid domains of the biomembrane [12, 31]. Assuming that a passive diffusion model is accurate, two properties of the anthracycline are important in determining their relative transport rates in a given cell line: (1) the overall membrane affinity, and (2) the pK_a of the amino sugar. These are considered in turn.

As demonstrated here and elsewhere [6, 7], increasing the hydrophobicity of the aglycone portion of an anthracycline through structural modification, which should not significantly affect the pK_a of the amino group, has been shown to significantly enhance anthracycline affinities for neutral and negatively charged membranes. Increased membrane affinity was generally found to result in increased net cellular uptake for adriamycin, daunomycin, and several other daunosamine-containing anthracyclines in a variety of cell lines including L1210, CCRF, HL-60, HeLa, and Ehrlich ascites (reviewed in [8]). Simply stated,

it is evident that if the drug has a higher probability of binding to a membrane, it also has a higher probability of diffusing through it when comparing congeners with equivalent pK_a.

Structural substitution of the anthracycline amino group, which dramatically alters pK_a, has been shown in this report to modulate drug binding to neutral and negatively charged membranes, particularly the latter. In addition, recent studies in the HL-60 leukemia cell line have shown that net cellular accumulation of anthracyclines is highly dependent upon substitution of the amino group, the steady-state drug levels for adriamycin and daunomycin derivatives decreasing in the order: *N,N*-dimethyl > morpholino > parent > cyanomorpholino [8]. This order is not consistent with equilibrium binding affinities but does parallel the available information on pK_a values. Thus, it appears that in a congeneric drug series the net cellular accumulation at the anthracyclines is primarily determined by the basicity of the amino group and that membrane affinity (i.e., relative hydrophilicity, hydrophobicity, or partition co-efficient) plays a secondary role.

Implications of anthracycline selectivity for negatively charged membranes in biological activity

Inhibition of malignant cell growth and cardiac toxicity are two of the major biological activities of the anthracyclines. The cardiac toxicity limits the therapeutic usefulness of these agents and may be the result of drug-induced free radical formation in mitochondria and sarcoplasmic reticulum [3, 16, 34]. Drug accumulation at these sites may be due to specific anthracycline-cardiolipin binding [13, 38]. The results of the present report demonstrate that the affinity of adriamycin and daunomycin for negatively charged membranes can be significantly reduced by substitution of the amino group.

The effects of anthracycline amino group substitution on cytotoxic potency against the HL-60 leukemia has recently been reported [8]. Soft agar clonogenic assays showed the following order of cytotoxicity for the adriamycin and daunomycin series: cyanomorpholino > parent > morpholino $\simeq$ *N,N*-dimethyl. Comparison of these potencies with the data contained in Table 2 indicate that anthracycline cytotoxicity is directly correlated with binding and inversely realted to pK_a for neutral model membranes. However, there is no qualitative correlation between cytotoxicity and negatively charged membrane affinity for these congeners. Thus, before a completely definitive assessment of the factors governing cellular membrane interaction is possible, information on drug partitioning among various membrane microdomains will be necessary.

Since it has been demonstrated that anthracyclines immobilized to various types of polymeric beads can kill tumor cells by a surface event [29, 30, 35, 37, 39], the notion of designing congeners which, in their native form, interact only with the cell surface is appealing. Two benefits to obtaining such drugs could be expected. First, these analogues would be useful in addressing the issue of whether anthracyclines can exert their activity through a surface mechanism similar to that of the non-penetrating polymer-immobilized forms of the drug. If such (a) surface mechanism(s) of antitumor activity is (are) available to anthracyclines in their native form, then targeting anthracyclines for the cell surface may result in the dissociation of antitumor activity from cardiotoxicity and mutagenicity, unwanted effects thought to be due to drug interaction at intracellular sites. The second benefit of surface-directed anthracyclines is the high cytotoxic potency demonstrated by such agents-often 1000-fold or more than the parent molecule [37].

One approach to the cell surface targeting of the anthracyclines is to design drugs which bind to membranes but do not accumulate intracellularly. We have demonstrated in this report that structural modifications in the aglycone portion of the drug molecule which enhance hydrophobicity also promote model membrane binding regardless of whether the membranes are neutral or negatively charged. In addition, we have demonstrated [8] that the net cellular accumulation of an anthracycline is dominated by the basicity of the amino group, derivatives with lower pK_a values exhibiting reduced uptake. Thus, the design of anthracyclines with hydrophobic aglycones and non-basic aminosugars appears to be a promising approach to the cell surface targeting of these antibiotics.

Acknowledgements. We are grateful to Dr Edward M. Acton of M.D. Anderson Hospital, Houston, Tex and Dr Derek Horton of The Ohio State University, Columbus, Ohio for contributing anthracyclines.

References

1. Acton EM (1980) *N*-Alkylation of anthracyclines. In: Crooke ST, Reich SD (eds) Antracyclines: Current status and new developments. Academic Press, New York
2. Acton EM, Tong GL, Mosher CW, Wolgemuth RL (1984) Intensely potent morpholinyl anthracyclines. J Med Chem 27: 638–645
3. Bachur NR, Gordon SL, Gee MV (1977) Anthracycline antibiotic augmentation of microsomal electron transport and free radical formation. Mol Pharmacol 13: 901–910
4. Barthelemy-Clavey V, Maurizot JC, Dimicoli JL, Sicard P (1974) Self-association of daunorubicin. FEBS Lett 46: 5–10
5. Burke TG, Tritton TR (1984) Ligand self-association at the surface of liposomes: a complication during equilibrium-binding studies. Anal Biochem 143: 135–140
6. Burke TG, Tritton TR (1985a) Structural basis of anthracycline selectivity for unilamellar phosphatidylcholine vesicles: an equilibrium-binding study. Biochemistry 24: 1768–1776
7. Burke TG, Tritton TR (1985b) Location and dynamics of anthracyclines bound to unilamellar phosphatidyl vesicles. Biochemistry 24: 5972–5980
8. Burke TG, Morin MJ, Sartorelli AC, Lane PE, Tritton TR (1987) Function of the anthracycline amine in membrane binding: Cellular transport and cytotoxicity. Mol Pharmacol 31: 552–556
9. Calendi E, DiMarco A, Reggiani M, Scarpinato B, Valentini L (1965) On physico-chemical interactions between daunomycin and nucleic acid. Biochim Biophys Acta 103: 25–49
10. Chaires JB, Dattagupta N, Crothers DM (1982) Self-association of daunomycin. Biochemistry 21: 3927–3932
11. Chang EL, Gaber BP, Sheridan JP (1982) Photon correlation spectroscopy study on the stability of small unilamellar DPPC vesicles. Biophys J 39: 197–201
12. Dalmark M, Storm HH (1981) A Fickian diffusion transport process with features of transport catalysis. Doxorubicin transport in human red blood cells. J Gen Physiol 78: 349–364
13. Duarte-Karim M, Ruysschaert JM, Hildebrand J (1976) Affinity of adriamycin to phospholipids: a possible explanation of cardiac mitochondrial lesions. Biochem Biophys Res Commun 71: 658–663

14. Gaber BP, Sheridan JP (1982) Kinetic and thermodynamic studies of the fusion of small unilamellar phospholipid vesicles. Biochim Biophys Acta 685: 87–93
15. Goldman R, Facchinetti T, Bach D, Raz A, Shinitzki M (1978) A differential interaction of daunomycin, adriamycin and their derivatives with human erythrocytes and phospholipid bilayers. Biochim Biophys Acta 512: 254–269
16. Goodman J, Hochstein P (1977) Generation of free radicals and lipid peroxidation by redox cycling of adriamycin and daunomycin. Biochem Biophys Res Commun 77: 797–803
17. Goormaghtigh E, Chatelain P, Caspers J, Ruysschaert JM (1980) Evidence of a specific complex between adriamycin and negatively charged phospholipids. Biochim Biophys Acta 597: 1–14
18. Graham I, Gagne J, Silvius JR (1985) Kinetics and thermodynamics of calcium-induced lateral phase separations in phosphatidic acid containing bilayers. Biochemistry 24: 7123–7131
19. Henry N, Fantine EO, Bolard J, Garnier-Suillerot A (1985) Interaction of adriamycin with negatively charged model membranes evidence of two types of binding sites. Biochemistry 24: 7085–7092
20. Kraczmar GS, Tritton TR (1979) The interaction of adriamycin with small unilamellar vesicle liposomes. A fluorescence study. Biochim Biophys Acta 557: 306–319
21. Kovaouci R, Silvius JR, Graham I, Pezolet M (1985) Calcium-induced lateral phase separations in phosphatidylcholine-phosphatidic acid mixtures. A Ramon spectroscopic study. Biochemistry 24: 7132–7140
22. Lenaz L, Page JA (1976) Cardiotoxicity of adriamycin and related anthracyclines. Cancer Treat Rev 3: 111–120
23. Martin SR (1980) Absorption and circular dichroic spectral studies on the self-association of daunorubicin. Biopolymers 19: 713–721
24. Menozzi M, Valentini L, Vannini E, Arcamone F (1984) Self-association of doxorubicin and related compounds in aqueous solutions. J Pharm Sci 73: 766–770
25. Mosher CW, Wu HY, Fujiwara AN, Acton EM (1982) Enhanced antitumor properties of 3'-(4-morpholinyl) and 3'-(4-methoxy-1-piperidinyl) derivatives of 3'-deaminodaunorubicin. J Med Chem 25: 18–24
26. Papahadjopoulos D, Hui S, Vail WJ, Poste G (1976a) Studies on membrane fusion: I. Interactions of pure phospholipid membranes and the effects of myristic acid, lysolecithin, proteins and dimethyl sulfoxide. Biochim Biophys Acta 448: 245–264
27. Papahadjopoulos D, Vail WJ, Pangborn WA, Poste G (1976b) Studies on membrane fusion: II. Introduction of fusion in pure phospholipid membranes by calcium ions and other divalent metals. Biochim Biophys Acta 448: 265–283
28. Poste GE, Papahadjopoulas D, Vail WJ (1976) Lipid vesicles as carriers for introducing biologically active materials into cells. Methods Cell Biol 14: 33–71
29. Rogers KE, Tokes ZA (1984) Novel mode of cytotoxicity obtained by coupling inactive anthracycline to a polymer. Biochem Pharmacol 33: 605–608
30. Rogers KE, Carr BI, Tokes ZA (1983) Cell surface-mediated cytotoxicity of polymer-bound adriamycin against drug resistant hepatocytes. Cancer Res 43: 2741–2748
31. Siegfreid JM, Burke TG, Tritton TR (1985) Cellular transport of anthracyclines by passive diffusion. Implications for drug resistance. Biochem Pharmacol 34: 593–598
32. Sturgeon RJ, Schulman SG (1977) Electronic absorption spectra and protolytic equilibria of doxorubicin: direct spectrophotometric determination of microconstants. J Pharm Sci 66: 958–961
33. Suurkuusk J, Lentz BR, Barenholz Y, Biltonen RL, Thompson TE (1976) A calorimetric and fluorescent probe study of gel-liquid crystalline phase transition in small, single-lamellar dipalmitoylphosphatidylcholine vesicles. Biochemistry 15: 1393–1401
34. Thayer WS (1977) Adriamycin stimulated superoxide formation in submitochondrial particles. Chem Biol Interact 19: 265–278
35. Tokes ZA, Rogers KE, Rembaum A (1982) Synthesis of adriamycin-coupled polyglutaraldehyde microspheres and evaluation of their cytostatic activity. Proc Natl Acad Sci USA 79: 2026–2030
36. Tong GL, Wu HY, Smith TH, Henry DW (1979) Adriamycin analogues: 3. Synthesis of *N*-alkylated anthracyclines with enhanced efficacy and reduced cardiotoxicity. J Med Chem 22: 912–918
37. Tritton TR, Yee G (1982) The anticancer agent adriamycin can be actively cytotoxic without entering cells. Science 217: 248–250
38. Tritton T, Murphree S, Sartorelli A (1978) Adriamycin: a proposal on the specificity of drug action. Biochem Biophys Res Commun 84: 802–808
39. Wingard LB Jr, Tritton TR, Egler KA (1985) Cell surface effects of adriamycin and corminomycin immobilized on cross-linked polyvinyl alcohol. Cancer Res 45: 3529–3536

Received April 16, 1987/Accepted November 30, 1987